THE JOURNAL OF INFECTIOUS DISEASES • VOL. 157, NO. 1 • JANUARY 1988
 0022-1899/88/5701-0007$01.00

Failure of Antisera to J5 and R595 Rough Mutants to Reduce Endotoxemic Lethality

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Rabbit antisera to J5 (Rc) *Escherichia coli* and R595 (Re) *Salmonella minnesota* rough mutants were selected for the highest content of hemagglutinating antibodies to their respective core glycolipids. Despite titers of 1:2560 vs. J5 and 1:640 vs. R595 core glycolipids, the antisera failed to passively protect ICR or CF-1 mice against lethality induced by endotoxins from a variety of wild-type, smooth enterobacteria: *E. coli* O111:B4, *E. coli* O127:B8, *Salmonella typhimurium*, *S. minnesota*, and *Citrobacter freundii*. J5 antisera, however, reduced lethality from J5 core glycolipid. In contrast, O-specific rabbit antisera were consistently protective against the lethal activity of wild-type, smooth enterobacterial endotoxins, but such protection was limited to the homologous endotoxin. These findings are consistent with in vitro demonstrations of a highly restricted ability of antibodies to J5 and R595 core glycolipids to bind to endotoxins from wild-type, smooth enterobacteria.

Antibodies with specificity for O-polysaccharide side chains of gram-negative bacterial endotoxins confer significant survival advantage in animal models of endotoxemia and of gram-negative bacterial sepsis [1–5]. This specificity, however, has hampered efforts to develop a "broad-spectrum" gram-negative bacterial vaccine because there are marked serological differences in O-polysaccharide structures not only between species, but also between most strains of the same species, of enterobacteria [6].

Because the structures of the core glycolipid and lipid A of endotoxins vary much less between different strains of enterobacteria [6], several laboratories have explored the potential of antisera to these antigens to confer broad-spectrum protection. Lipid A–specific antisera have not been found to protect normal animals against wild-type, smooth enterobacterial infections or their endotoxins [7–9]. Core-specific antisera, however, have been reported to passively confer broad-spectrum protection against both types of challenge [8–18]. Strains of rough mutant bacteria, in particular the J5 (Rc) mutant of *Escherichia coli* O111:B4 and the R595 (Re) mutant of *Salmonella minnesota*, which lack O-polysaccharide side chains otherwise masking the core glycolipid, have been used to elicit these antisera. It has been postulated that the broad-spectrum protective activity of these antisera is mediated by the core-specific antibodies [8–18].

We have previously examined the protection provided by J5 and R595 rough mutant rabbit antisera in murine models of gram-negative bacterial infections, both with and without antibiotic treatment [3, 19]. In contrast to results by some investigators [8–15], when matched preimmune serum controls from the same donors were used, no protection attributable to common core-specific antibodies could be demonstrated [3, 19]. These negative findings have been confirmed by others [5, 20–24]. O-specific antisera were, however, consistently protective.

Herein, we report the inability of rabbit antisera to the rough mutants J5 and R595, selected for the highest content of HA antibodies to their respective core glycolipids, to protect mice against the lethal activity of endotoxins from a variety of wild-type, smooth enterobacteria. This contrasts with the consistent and highly significant protection against endotoxin lethality provided by O-specific antisera and parallels our earlier findings with the infection models.

Received for publication 23 April 1987, and in revised form 10 August 1987.

This study was presented at the annual meeting of the American Society for Clinical Investigation held on 7 May 1984 in Washington, D.C.

These studies were supported by a grant from the Frank C. Bressler Fund, University of Maryland School of Medicine.

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Materials and Methods

Animals. Outbred ICR male mice, weighing 20–25 g, were obtained from Dominion Laborato-

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ries (Dublin, VA). They were housed 10 per cage, fed Purina Rodent Chow (Ralston-Purina, Richmond, Ind) and tap water ad libidum, and acclimatized for five to seven days before each experiment.

Rabbits, used to prepare antisera, were New Zealand albinos, weighing 2–3 kg, of mixed sexes and were obtained from a single supplier. These rabbits had never been injected with any agent and had been healthy since birth.

Endotoxins and rough mutant bacteria. Endotoxins (lipopolysaccharides or LPS) from all wild-type, smooth *E. coli* and *Salmonella* strains were Boivin preparations from Difco Laboratories (Detroit, Mich) or Sigma Chemical (St. Louis). A highly purified phenol-water–extracted and electrodialyzed LPS from a smooth strain of *Citrobacter freundii*, which was converted to the uniform triethylamine salt form and free of proteins or nucleic acids, was provided by Dr. Helmut Brade (Institut für Experimental Biologie und Medizin, Borstel, FRG). Purified J5 and R595 core glycolipids were obtained from several different laboratories; these are considered further in the Results. Rough mutant *S. minnesota* R595, chemotype Re, was obtained from Dr. Siegfried Schlech (Max Planck Institut für Immunbiologie, Freiburg, FRG). The J5 rough mutant of *E. coli* O111:B4, chemotype Rc, was obtained from Dr. Edward Heath (University of Iowa, Iowa City) via Rhona Stein (University of Pittsburgh, Pittsburgh). Nonviable, dried preparations of the J5 and R595 rough mutants were provided by Dr. Linda Shoer (List Biologicals, Campbell, Calif). These preparations had been washed with distilled water after harvesting; dried by sequential treatment with 95% ethanol, acetone, and petroleum ether; and then air-dried at room temperature until no ether vapors could be detected.

Preparing antisera. O-specific antisera were obtained by daily iv injections of rabbits with Boivin-extracted LPS from wild-type, smooth enterobacteria in doses of 0.05, 0.5, 5.0, 5.0, 5.0, and 5.0 µg/kg. Serum was obtained seven days after the last injection. This immunization schedule elicited antibody titers to the respective smooth LPS comparable to titers produced to core glycolipid endotoxins when whole rough mutants were used as vaccines (see below). O-specific antiserum was also prepared by injecting the smooth parental strain of J5 (*E. coli* O111:B4) and by using the same procedures outlined below for rough mutant antisera. This O-specific antiserum was used for direct comparisons with the protective activity of the J5 mutant antisera.

To prepare antisera to the J5 and R595 rough mutant endotoxins, we used whole bacteria, because the purified core glycolipid endotoxins of these mutants were much less immunogenic than the Boivin-prepared LPS from smooth strains. Six to 10 typical J5 or R595 colonies were selected from blood agar plates and collectively grown for 18–20 h at 37 C in trypticase-soy broth, free of galactose. The broth cultures were washed three times in sterile pyrogen-free 0.9% NaCl, heated to 100 C for 10 min, and suspended in the saline at a concentration of $\sim 10^8$/mL. Rabbits were injected iv with 1.0-mL aliquots of the bacterial suspensions for three consecutive days in each of two weeks. Serum was obtained seven days after the last (sixth) dose of antigen. The dried, nonviable J5 and R595 rough mutants obtained from List Biologicals were suspended in pyrogen-free, sterile 0.9% NaCl; washed three times with the saline; and injected sequentially as described above, but $\sim 1.25 \times 10^8$ organisms were used for the initial two injections, 2.5×10^8 for the next two injections, and 5×10^8 for the final two injections to produce additional aliquots of rough mutant antisera. Antiserum to the J5 *E. coli* mutant was also prepared according to the method of Braude et al. [25]: overnight broth cultures were washed three times in sterile, pyrogen-free saline, boiled for 2.5 h (rather than 10 min), and resuspended to a density allowing 70% light transmission at 610 nm. One-milliliter aliquots were injected iv, and the rabbits were bled seven days after the last of six injections of the boiled cells that were given three times weekly for two weeks [25]. The various preparations of rough mutant antisera used for testing are summarized in table 1.

For control purposes, preimmune serum from the corresponding donors was obtained five to seven days before immunization by bleeding 10 mL from the central ear artery and was pooled in the same proportions as the immune sera. Except where specified, each pool represents serum from three to five animals. All antisera and preimmune sera were drawn by using a sterile and pyrogen-free technique and were stored in aliquots in pyrogen-free, sterile containers at −20 C for a least two weeks before the protection studies in mice. Because serum given to heterologous species can be toxic and because such foreign serum toxicity can be reduced by heating [26], both heated (56 C for 30 min) and unheated aliquots

Table 1. Rough mutant antisera.

Serum	Source of bacteria	Treatment of bacteria	Homologous HA titer of antiserum to LPS
R595, pool 1	Schlecht	Heat-killed (10 min)	1:640
R595, pool 2	List Biologicals	Ethanol, acetone, petroleum-ether	1:640
J5, pool 1	Heath	Heat-killed (10 min)	1:2560
J5, pool 2	Heath	Heat-killed (10 min)	1:2560
J5, pool 3	List Biologicals	Ethanol, acetone, petroleum-ether	1:2560
J5, rabbit 25	Heath	Heat-killed (2.5 h)	1:2560
J5, rabbit 26	Heath	Heat-killed (2.5 h)	1:2560

NOTE. All antisera were prepared by administering three iv injections of bacteria daily for two weeks, followed by bleeding seven days after the last injection (see Materials and Methods).

of these stored pairs of sera were tested in most studies. No differences in protection were observed, and the results have therefore been combined.

Lethal model. Before each study, dose-response lethality curves were determined for each wild-type LPS by iv injection of 0.5 mL of twofold dilutions in sterile, pyrogen-free saline by using groups of five to 10 mice per dilution. The J5 endotoxin was titrated similarly after compensating for its insolubility by suspension in sterile, pyrogen-free water; heating at 100 C for 5 min; sonicating; and then diluting 1:9 in sterile, pyrogen-free 0.9% NaCl. Cumulative mortality was recorded at 96 h, because all deaths occurred within this period. Challenge doses of LPS, used for evaluating protection by antisera, were always kept within the sensitive dose-response range, between one and two LD_{50}. Protective activity of antisera was assessed by administering the indicated volume iv 30–60 min before LPS challenge. Differences in numbers of animals receiving serum within each individual study are based upon differences in amounts of serum collected and not upon retrospective addition or exclusion of test results.

Antibody titrations. To ensure that the core glycolipid antigens in the J5 and R595 rough mutants used for immunization were comparable to those used by other investigators, we titered the resultant antisera against purified core glycolipids obtained from other laboratories, rather than those extracted from the present immunizing cultures. J5 core glycolipid, extracted by the phenol-water technique, was provided by the late Abraham Braude, (University of California, San Diego). R595 core glycolipid, extracted with phenol-chloroform–petroleum ether, was provided by Otto Luderitz (Max Planck Institut für Immunbiologie). These rough mutant endotoxins were treated with sodium hydroxide, as previously described, to permit adsorption to human O red blood cells [27] and to bentonite [3].

The rough mutant antisera were titered by using both the passive HA and bentonite agglutination (BA) techniques [27, 28]. By both methods, antibody titers to the homologous core glycolipids ranged between 1:160 and 1:2560. Those antisera with the highest titers to the core glycolipid were selected for passive protection studies. These antisera were then further screened for increments in O-specific antibodies to the challenge LPS preparations that might have resulted from polyclonal B cell stimulation (see below). Detectable increments in HA and BA titers (>1:10) were found in some sera, but these were limited to the parental LPS; such sera were excluded. All preimmune sera possessed HA and BA antibody titers <1:10 to the respective rough mutant core glycolipid preparations.

The antisera to LPS from wild-type, smooth bacterial strains possessed HA and BA titers of 1:320–1:640 to the homologous LPS. The antiserum to the boiled *E. coli* O111:B4 possessed an HA titer of 1:2560 against the homologous LPS. All O-specific antisera exhibited titers <1:10 to the heterologous LPS used for challenge.

Statistical analysis. Because each experiment usually involved comparisons between more than two groups, statistical significance was determined by using the χ^2 test for homogeneity of samples from binomially distributed populations [29]. This method assures that the χ^2 test is not simply applied to arbitrarily selected pairs of results, but instead takes into account the phenomenon of multiplicity, i.e., the increasing probability that significant differences between any two groups will occur merely as the result of chance as the number of experimental groups increases.

Results

O-specificity of protection. Rabbit antisera to *S. typimurium* LPS and to *E. coli* O111:B4 LPS conferred significant protection to ICR mice against challenge with 2 LD_{50} of LPS, but this result was restricted to the homologous LPS (table 2). Rabbit antiserum to an unrelated antigen, human serum albumin, was not protective. Of interest, the antiserum lost its effectiveness when administered 30 min after injecting the homologous LPS.

Strain specificity of passive protection is shown in table 3. Antisera to *E. coli* O127:B8 LPS protected ICR mice against challenge with 2 LD_{50} of LPS, but such protection was limited to the LPS from the homologous *E. coli* serotype. Antiserum to *E. coli* O111:B4 LPS also appeared to be protective only against the lethal activity of LPS from the homologous *E. coli* serotype, although such protection was not statistically significant.

Lack of protection by rough mutant antisera. The protective activity of rabbit antiserum to the R595 *S. minnesota* rough mutant (R595 pool 1), possessing an HA titer of 1:640 to R595 core glycolipid, was tested against lethality induced by one LD_{50} of LPS from smooth *S. minnesota* (the parental strain), and 2 LD_{50} of *E. coli* O127:B8 LPS (table 4). Mortality of ICR mice treated with the R595 antiserum was not lower than that of controls treated with saline or matched, preimmune pooled serum. In contrast, antiserum to *E. coli* O127:B8 LPS was highly protective, although again only for the homologous LPS.

Antiserum to the J5 (Rc) *E. coli* rough mutant, possessing an HA titer of 1:2560 to J5 core glycolipid, was tested for protective activity against 2 LD_{50} of LPS from *E. coli* O111:B4 (the parental strain) and *S. typhimurium* (table 5). The J5 antiserum (J5 pool 1) had no protective effect in ICR mice against lethality of either LPS, compared with saline or matched, preimmune pooled serum. In contrast, *E. coli* O111:B4 antiserum was protective for the homologous LPS.

The above experiment was repeated with a pool of J5 antiserum (J5 pool 2) drawn from another group of rabbits possessing an HA titer of 1:2560 to J5 core glycolipid, as well as with the original R595 antiserum (R595 pool 1), by using one, rather than two, LD_{50} quantities of *E. coli* O111:B4 LPS (table 6). Again, the J5 and R595 rough mutant antisera had no protective effect in ICR mice, compared with saline, whereas the homologous O-specific antiserum was highly protective.

Table 2. Species specificity of protection by O-antisera.

Treatment	Percent mortality (n): *S. typhimurium* LPS (500 µg)	Percent mortality (n): *E. coli* O111:B4 LPS (300 µg)
Pretreatment (0.5 mL)		
Saline	100 (10)	73 (11)
Antiserum to *S. typhimurium*	36 (28)*	83 (30)
Antiserum to *E. coli* O111:B4	100 (20)	38 (32)†
Antiserum to human serum albumin	100 (10)	
30-min posttreatment (0.5 mL)		
Antiserum to *S. typhimurium*	100 (10)	

* $P < .0005$, compared with the other *S. typhimurium* LPS trials.
† $P < .005$, compared with the other *E. coli* LPS trials.

Table 3. Strain specificity of protection by O-antisera.

Pretreatment (0.25 mL)	Percent mortality (n): *E. coli* O127:B8 LPS (500 µg)	Percent mortality (n): E. coli O111:B4 LPS (300 µg)
Saline	100 (10)	83 (12)
Antiserum to *E. coli* O111:B4	90 (10)	57 (14)*
Antiserum to *E. coli* O127:B8	0 (10)†	100 (12)

* $P < .15$, compared with the other O111:B4 LPS trials.
† $P < .0005$, compared with the other O127:B8 LPS trials.

Table 4. Lack of protection by R595 antiserum.

Pretreatment	Percent mortality (n): *S. minnesota* LPS (500 µg)	Percent mortality (n): *E. coli* O127:B8 LPS (500 µg)
0.5 mL		
Saline	58 (12)	93 (15)
Antiserum to R595*	60 (10)	93 (15)
Preimmune serum*	QNS†	93 (15)
0.25 mL		
Antiserum to *E. coli* O127:B8	69 (13)	27 (15)‡
Preimmune serum	58 (12)	100 (15)

* R595 pool 1.
† QNS, serum quantity not sufficient for study.
‡ $P < .0005$, compared with the other *E. coli* LPS trials.

Table 5. Lack of protection by J5 antiserum.

Pretreatment (0.5 mL)	Percent mortality (n) *E. coli* O111:B4 LPS (300 μg)	*S. typhimurium* LPS (500 μg)
Saline	88 (17)	100 (10)
Antiserum to J5*	100 (10)	100 (15)
Preimmune serum*	100 (10)	93 (15)
Antiserum to *E. coli* O111:B4	33 (12)†	NT‡

* J5 pool 1.
† $P < .01$, compared with the other O111:B4 LPS trials.
‡ NT, not tested.

Table 7. Lack of protection by J5 antisera prepared by using the method of Braude et al. [25].

Pretreatment (0.5 mL)	Percent mortality (n) with *S. typhimurium* LPS (230 μg)
Saline	52 (25)
Antiserum to J5*	75 (12)
Preimmune serum*	64 (11)
Antiserum to J5†	83 (12)
Preimmune serum†	83 (12)
Antiserum to *S. typhimurium*	0 (25)‡

* Serum from rabbit 25 (not previously tested).
† Serum from rabbit 26 (not previously tested).
‡ $P < .0005$, compared with the other trials.

Because previous studies claiming the efficacy of rough mutant antisera for protection against endotoxin lethality used the CF-1 mouse [16–18], the antisera to J5 (J5 pool 2) and R595 (R595 pool 1) were also tested in this murine strain. One LD_{50} of *E. coli* O111:B4 LPS was used for challenge (table 6). Again, the percent mortality obtained with these antisera was not lower than the percent mortality in saline-treated mice. In contrast, homologous O-specific antiserum was again highly protective.

The efficacy of two individual samples of J5 rabbit antisera, which were prepared strictly according to the method of Braude and co-workers [25] and which possessed HA titers of 1:2560 to J5 core glycolipid, was also assessed (table 7). No evidence of protective activity was seen, compared with either preimmune serum or saline, against lethality induced in ICR mice by one LD_{50} of *S. typhimurium* LPS. In contrast, homologous O-specific antiserum was highly protective.

The efficacy of R595 and J5 rabbit antisera, prepared by using dried, nonviable rough mutants obtained from List Biologicals, was also tested. As in the previous studies, for each rough mutant, those antisera containing the highest HA antibody titers (1:640 and 1:2560 to the R595 and J5 core glycolipids, respectively) were pooled (R595 pool 2 and J5 pool 3). Again, no significant protective activity against challenge with one LD_{50} of *S. typhimurium* LPS was seen in ICR mice with either antiserum (table 8).

Table 6. Lack of protection by J5 and R595 antisera.

Pretreatment (0.5 mL)	Percent mortality (n) with *E. coli* O111:B4 LPS* in ICR mice	CF-1 mice
Saline	52 (29)	50 (20)
Antiserum to J5†	67 (18)	50 (20)
Antiserum to R595‡	70 (20)	50 (20)
Antiserum to *E. coli* O111:B4	0 (21)§	5 (20)‖

* LPS dose, 175 μg for ICR mice and 220 μg for CF-1 mice.
† J5 pool 2.
‡ R595 pool 1.
§ $P < .0005$, compared with the other ICR trials.
‖ $P < .01$, compared with the other CF-1 trials.

The ability of J5 antiserum (J5 pool 2) to protect against one LD_{50} of a highly purified phenol-water preparation of LPS from smooth *Citrobacter freundii* (200 μg), electrodialyzed and converted to the uniform triethylamine salt form, was also tested. No protective activity in ICR mice was discernible (J5 antiserum, 50% mortality [n = 10]; preimmune sera, 50% mortality [n = 10]).

The ability of J5 antiserum (J5 pool 2) to reduce mortality from one LD_{50} of a phenol-water–extracted J5 core glycolipid endotoxin, obtained from List Biologicals, was also tested in ICR mice. In contrast to its consistent ineffectiveness against wild-type LPS, the J5 antiserum was now significantly protective when compared with preimmune serum or with pooled normal serum obtained from other healthy donors (table 9).

Because some studies claiming the broad-spectrum efficacy of J5 and R595 rabbit antisera to LPS used mice sensitized to LPS lethality by actinomycin D [18], we repeated the above studies by using this model. Groups of 10 ICR mice each were given 0.5 mL of preimmune, J5 (pool 1), or R595 (pool 1) rabbit antiserum iv and 1 h later were challenged iv with 2 LD_{50} of *E. coli* O127:B8 LPS (0.25 μg) mixed with 15 μg of actinomycin D. As in the previous studies, these rough mutant antisera had been screened to ensure that no detectable increments

Table 8. Lack of protection by J5 and R595 antisera, prepared by using dried, nonviable organisms.

Pretreatment (0.5 mL)	Percent mortality (*n*) with *S. typhimurium* LPS (250 μg)
Saline	33 (21)
Antiserum to R595*	47 (19)
Preimmune serum	63 (8)
Antiserum to J5†	50 (20)
Preimmune serum	42 (19)

* R595 pool 2.
† J5 pool 3.

Table 9. Efficacy of J5 antiserum against J5 endotoxin lethality.

Pretreatment (0.5 mL)	Percent mortality (*n*) with *E. coli* J5 glycolipid (13–14 μg/g)
Antiserum to J5*	15 (27)†
Preimmune serum	78 (9)
Normal rabbit serum	62 (26)

* J5 pool 2.
† $P < .001$, compared with the other trials.

(<1:10) in HA titers to the challenge LPS had been evoked as a consequence of polyclonal B cell stimulation during immunization. Despite the ~2000-fold reduction in LD_{50} of the LPS elicited by the actinomycin D and testing within the most sensitive dose-response range, the J5 and R595 antisera again failed to confer any protection (data not shown).

Effects of normal rabbit serum. In all the present studies, the rabbit sera had been stored for two or more weeks at −20 C. The effect of individual normal rabbit sera, stored for similar periods, on resistance of mice to LPS was therefore examined (table 10).

The data suggest that although 0.5 mL of stored normal rabbit serum often has no significant effect compared with pyrogen-free, sterile saline, enhancement of LPS lethality may occur. Enhancement is also suggested by data in tables 6 and 7. Because of such variable effects of different normal rabbit sera on LPS lethality, the present control studies for the J5 and R595 antisera used both saline and aliquots of matched, preimmune serum obtained from the same donors and collected and pooled in identical fashion as the immune serum.

Discussion

The present findings demonstrate that high-titered J5 and R595 rabbit antisera failed to protect mice against lethality produced by LPS from heterologous, smooth enterobacteria or even from the homologous, smooth parental strain. In contrast, O-specific antisera were consistently protective against the homologous, smooth LPS. These results do not confirm those reported by previous investigators [16–18] and do not support their hypothesis that antibodies to the core glycolipids of J5 and R595 are capable of effective, broad-spectrum neutralization of the lethal activity of smooth, wild-type bacterial LPS.

Our inability to demonstrate broad-spectrum protection against LPS lethality with J5 and R595 rough mutant antisera cannot be attributed to excessive test doses of LPS. Challenge doses between one and two LD_{50} were used. These are comparable to those used by others reporting protection [16–18] and fall within the most sensitive portion of the dose-response curve for each LPS. Moreover, these test doses allowed the demonstration of highly significant and specific protection by O-specific antisera.

Likewise, the lack of broad-spectrum protection by the rough mutant antisera cannot be attributed to inadequate volumes or to inadequate antibody titers to the J5 or R595 core glycolipids. In the present studies, only the highest titered antisera were used, and 0.5-mL aliquots consistently failed to reduce LPS lethality. Yet, Johns et al. [18] reported highly significant protection with 0.3 mL of R595 rabbit antiserum containing half the present HA an-

Table 10. Effect of normal rabbit serum on endotoxin lethality.

Pretreatment (0.5 mL)	Percent mortality (*n*) with *E. coli* O111:B4 LPS: 140 μg	220 μg
ICR mice		
Saline	42 (29)*	
NRS, A	25 (20)	
NRS, B	25 (20)	
NRS, C	35 (20)	
NRS, D	47 (17)	
NRS, E	50 (20)	
CF-1 mice		
Saline		50 (20)
NRS, F		95 (20)†

NOTE. NRS = normal rabbit serum. A–F = different individual rabbit sera.
* $P > .7$, compared with the trials in ICR mice.
† $P < .005$, compared with the saline control.

tibody titers to the core glycolipid, and even 0.2 mL conferred protection. Moreover, in the present studies, 0.25 mL of O-specific antisera was highly protective despite antibody titers to the homologous, smooth LPS comparable to the antibody titers to the core glycolipid in the rough mutant antisera.

Differences in the strain of mice also could not account for the divergent results. The CF-1 mouse was used by those investigators reporting broad-spectrum protection against LPS lethality with J5 and R595 rabbit antisera [16–18]. Repeat studies in CF-1, rather than ICR, mice were therefore performed. These studies also failed to show protection against wild-type LPS, whereas highly significant protection was again seen with homologous O-specific antisera.

Differences in the method of preparation of the rough mutant antisera might have accounted for the divergent results. Hence, the method reported by Braude and co-workers [25], involving boiling of the J5 rough mutant for 2.5 h and immunizing rabbits over a two-week period, was duplicated. Again, the resulting J5 antiserum was not protective against a heterologous, smooth enterobacterial LPS. Because McCabe et al. [18] used acetone-killed bacilli as a vaccine and used progressively increasing doses, we also tested rabbit antisera against J5 and R595 rough mutant vaccines whose preparation included acetone treatment and which were given in increasing amounts; these too provided no broad-spectrum protection. Heat-inactivation of the rabbit antisera at 56 C for 30 min, performed to reduce possible foreign serum toxicity, also could not account for failure of protection, because unheated serum also failed to protect and because the protection by J5 and R595 rabbit antisera reported by McCabe et al. [18] was assessed by using heat-inactivated sera. Differences in the interval between completion of immunization and serum collection also could not account for failure of protection by the J5 and R595 rabbit antisera because the same interval was used by those reporting protection [16–18].

The possibility that the core glycolipid antigens in the present J5 and R595 mutants were not comparable to those of other investigators was also considered. This was evaluated in the following two ways: (*1*) the present antisera were titered against the J5 and R595 core glycolipids supplied by other investigators and shown to possess HA titers to these glycolipids comparable to those of others claiming protection, and (*2*) each of the J5 and R595 rough mutants used as vaccines was obtained from two different laboratories; the different pools of antisera gave identical results.

That differences in the methods of preparation of the challenge LPS might account for the divergent findings was considered. We used Boivin preparations; however, these preparations were also used by those reporting protection by the rough mutant antisera [16, 17]. McCabe et al. [18] used LPS from phenol-water or phenol-chloroform–petroleum ether extracts, which were electrodialyzed, scraped from membranes, and solubilized with triethylamine. We therefore also performed studies with a similar preparation from a smooth enterobacterium. Again, no protection by J5 rough mutant antiserum was observed.

In one study in which R595 rabbit antiserum was reported to provide broad-spectrum protection against LPS, actinomycin D was used to sensitize the mice to LPS lethality [18]. We therefore also performed studies using this model. As with normal mice, no protection by J5 or R595 rabbit antisera was discernible against wild-type LPS.

Thus, the explanation for the discrepancy in the findings of the ability of antisera to J5 and R595 rough mutants to confer broad-spectrum protection against LPS lethality is not readily apparent. Several explanations are conceivable. One is that the broad-spectrum protection reported by others, in fact, results from polyclonal increments in O-specific antibodies. As we demonstrated and as reviewed exhaustively elsewhere [1], it is clear that O-specific antisera uniformly provide significant protection against lethality induced by endotoxins from homologous, smooth bacterial strains. Endotoxins from rough mutants can induce antibodies with specificity for diverse O-polysaccharides through their ability to polyclonally activate B lymphocytes [30]. In addition, biochemical observations indicate that some rough mutants defective in the synthesis of core polysaccharides (rfa mutants) produce O-polysaccharide chains that cannot be attached to the LPS core [31–33]. Several investigators have demonstrated especially significant rises in titers of serotype-specific antibodies to the parental smooth LPS in animals immunized with rough mutant organisms [3, 30, 34–37]. This effect was also seen in our studies. Thus, it is possible that O-polysaccharides not expressed on the surface of the intact living mutant may become immunogenic during preparation of the killed bacterial vaccine. Alternatively, reversion of one of

the multiplying mutants to the parental form could lead to mixtures of O-polysaccharide and "unencumbered" core glycolipid in the vaccine. Therefore, in our studies, all rough mutant antisera, screened for their highest content of antibodies to core glycolipid, were further screened for increments in agglutinating antibodies to the wild-type, smooth endotoxins used for testing. None of these antibodies were detectable to the challenge heterologous wild-type LPS, but sera with substantial (>1:10) increments to the homologous parental LPS were occasionally detected; these were expressly excluded. Because no such precautions were specified in most previous studies, the observed broad-spectrum protection against LPS lethality by rough mutant antisera could be based upon polyclonal increments in O-specific antibodies. Indeed, because polyclonal increments in O-specific antibodies appear to be more readily evoked by rough mutant than by wild-type, smooth enterobacterial strains [30], rough mutant antisera would be more likely than O-specific antisera to be broadly protective by this mechanism.

Another explanation for the divergent findings regarding the broad-spectrum protection by rough mutant antisera may reside in the varying ability of foreign sera to enhance LPS lethality. Striking enhancing effects have been shown for normal human, guinea pig, and rat sera in rabbits [38], and our studies demonstrate occasional enhancing effects of normal rabbit serum in mice. For this reason, if normal sera possessing enhancing activity were inadvertently used as a control, those rough mutant antisera lacking such enhancing properties would appear to possess broad-spectrum protective activity, when, in fact, this activity was not present. To preclude such erroneous conclusions, we compared rough mutant antisera with matched, preimmune sera that were from the same donors and were collected and stored under comparable conditions, as well as with saline-treated controls. These critical controls were also apparently not used in most previous studies.

An additional explanation for the divergent findings regarding broad-spectrum protection against LPS by rough mutant antisera may relate to differences in LPS-neutralizing activity by acute-phase serum factors that modify LPS binding to serum lipoproteins. Thus, a Limulus lysate gelation inhibition assay showed LPS-neutralizing activity by J5 and R595 rabbit antisera to be increased in vitro, but this result could not be correlated with antibody to the rough LPS [39]. Recent studies have also shown that certain acute-phase serum proteins (e.g., α_2 macroglobulin) protect rats against endotoxin shock [40]. These mechanisms may explain the earlier conclusion from Braude's laboratory that O-specific rabbit antisera protected mice against lethality from heterologous, wild-type LPS as effectively as it does against homologous LPS [16, 17]. No such broad-spectrum protection by O-specific rabbit antiserum was seen in the present studies, nor in the studies by McCabe et al. [18]. The fact that the discordant observations with respect to broad-spectrum protection by rough mutant antisera extends to O-specific antisera further suggests involvement of factors other than antibodies to the inner core glycolipids in such protection.

A number of prior in vivo studies are consistent with the present findings. Thus, we observed over a decade ago that large quantities of rabbit J5 antiserum (10 mL/kg) conferred only minimal protection to rabbits against fever induced by trace amounts (10 ng/kg) of a heterologous, smooth LPS [34]. No protection against LPS-induced fever was observed by Milner [41] with rabbit antisera to the Re chemotypes of *S. minnesota* or *S. typhimurium*. Protection against LPS-induced fever could not be obtained by Ralovich et al. [42], despite a 1-h preincubation at 37 C of Boivin-extracted *E. coli* O83 or *Salmonella enteritidis* LPS with R595 rabbit antiserum. In contrast, O-specific antiserum was highly protective against LPS pyrogenicity [34, 41]. Equine J5 antiserum failed to protect horses against challenge with the parental wild-type *E. coli* LPS [43]. J5 antiserum did appear to protect sheep against challenge with *Serratia marcescens* LPS [44]; however, the J5 antiserum was obtained from donor sheep that had been challenged with the *S. marcescens* LPS one week previously, an action thereby invalidating meaningful conclusions. Sakulramrung and Domingue [45] reported that 1 mL of high-titered rabbit J5 antiserum failed to protect mice against challenge with various quantities of *S. typhimurium* endotoxin; in contrast, homologous antiserum to *S. typhimurium* afforded "full" protection. The authors concluded, "We do not have a plausible explanation for the failure of our antiserum to strain J5 to protect mice against lethal doses of endotoxin" [45]. We suggest that these findings, which are in complete agreement with the present results, can be related to the documented inability of antibodies to J5 core glycolipids to bind to wild-type, smooth LPS [30, 46–53]. Such failure of binding was

also seen in the present studies because the rabbit J5 and R595 antisera failed to agglutinate erythrocytes or bentonite coated with LPS from heterologous, wild-type, smooth enterobacteria, whereas these same antisera, even diluted several hundred- or thousandfold, consistently agglutinated such particles coated with the respective core glycolipids.

That restricted ability of antibodies to core glycolipids to bind to wild-type, smooth LPS does indeed account for their ineffective protection and that this ability is based upon a "masking" effect of the O-specific polysaccharide side chains, as has been previously suggested [46–48, 53], were clearly demonstrated in the present studies by testing the ability of J5 rough mutant antiserum to reduce lethality from the homologous core glycolipid lacking the "covering" O-polysaccharide side chains. The same pool of J5 antisera that failed to protect against the parental smooth LPS was found capable of significantly reducing lethality from the "unencumbered" J5 core glycolipid. Moreover, such protection was as marked as that conveyed by O-specific antisera against its homologous, wild-type, smooth LPS.

The present findings raise serious questions regarding the hypothesis that the reduction of mortality by J5 antiserum reported in human gram-negative bacterial sepsis is based upon broad-spectrum LPS-neutralizing activity of antibodies to J5 core glycolipid [54, 55]. Thus, we could obtain no evidence for effective broad-spectrum neutralization of LPS lethality. Moreover, in a number of critically controlled experimental studies, J5 and R595 antisera failed to significantly lower mortality in both antibiotic- and nonantibiotic-treated experimental models of gram-negative bacterial sepsis, whereas O-specific antisera were consistently protective [3, 5, 19–24]. In addition, significant titers of "natural" O-specific antibodies to LPS exist in most human sera [4, 56–58]; these may greatly exceed those antibodies to the core glycolipid in the J5 antisera clinical trials [54]. Consequently, even though paired pre- and post-J5 immune human sera from the same donors were given in one clinical trial [54], valid conclusions regarding a protective role of core-specific antibodies would have been possible only if the protective effects of "natural" O-specific antibodies had been critically controlled by testing each set of paired sera against infections caused by identical bacterial serotypes. Even then, it would have also been necessary to carefully exclude the protective effects of polyclonal increments in O-specific antibodies induced by the J5 immunization, as well as any protective effects of acute-phase serum factors, before core-specific antibodies were credited for the protective activity of the antiserum. The importance of limiting comparisons of protection by any given set of pre- and postimmune serum to identical bacterial serotypes for analysis of protective mechanisms is further emphasized by the finding that antibodies to pneumococci, which may have been present in some of the donor sera, can cross-react with enterobacterial surface antigens (*Pseudomonas aeruginosa* LPS and K antigens of *E. coli* and *Klebsiella*) and cross-protect against enterobacterial sepsis [59, 60]. These considerations could explain the absence of any relation between enhanced survival and antibody titer to J5 core glycolipid in one clinical trial [54]. Failure to provide any antibody data throughout another clinical trial makes the conclusion that antibodies to J5 core glycolipid appear to protect by neutralizing LPS subject to question [55]. Moreover, in a third clinical trial in which pre- and postimmune J5 antisera were given prophylactically in comparable quantities to patients with neutropenia, no protection was conferred against gram-negative bacterial sepsis; in particular there was no difference in risk of bacteremia, febrile episodes, or mortality rates [61]. Because, in contrast to J5 and R595 antisera, O-specific antisera proved capable of significantly protecting against lethality from wild-type LPS, the present findings directly support the work of Gaffin and co-workers [57], who advocate pooled concentrates of normal human immunoglobulins containing mixtures of serotype-specific antibodies to LPS for a broad-spectrum immunological approach to treating endotoxemia during gram-negative bacterial infections.

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